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Issues in Biochemical Applications to Risk Assessment: Can *In Vitro* Studies Assist Us in Species Extrapolation?

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Introduction

My topic today is, Can *in vitro* studies assist us in species extrapolation? And I'm going to narrow this topic a little bit simply by concentrating on human data, thinking that is the species that we're most interested in. I would like to redefine the question and ask, Can *in vitro* studies or *in vivo-in vitro* studies be used to assess human genotoxicity?

I'd like to start with a quote from Dr. James Miller and just read this for the group:

> I do not think we can rely on extrapolation of data on chemical carcinogens from experimental animnals to humans no matter how sophisticated or plausible these extrapolations may seem, until we know more about the chemical carcinogenesis in humans.

There are a lot of ways to take this idea as the springboard for leading into the discussion, but I think one of the most direct ways is to try to glean what we can from the use of human cells in the short-term assays and try to make species extrapolations based on the short-term *in vitro* studies that one can do with human cells. So the question that I'd like to address instead of just, can *in vitro* studies be useful for risk assessment, is to ask, Are human cells going to be useful for risk assessment purposes?

I have an idea based on some of my own research and research from many other laboratories that the answer to that question might be yes. But today I will point out the research that has been done and the rationale for the types of studies that have been done so far.

This is a very timely topic, the use of human cells for carcinogenesis studies, as the January 1 issue of *Cancer Research* indicated. The cover showed Curtis Harris and his collaborators, pioneers in the use of human cells and tissues in biomedical research (*1*). The protocol which he uses, and basically all the other laboratories that try to use human cells for carcinogenesis research use, is to take whole animals and determine whether compound X will induce tumors, and if so, at what sites? Then take the sites where the compound X induced tumors and use tissues or isolated cells from those tissues in a short-term assay to determine whether compound X induces mutations, DNA damage, DNA repair, or any of a number of other end points for short-term assays, such as induction of aneuploidy.

One can take all of the short-term tests that can be done to assess genotoxicity, take the tissues from the rodents or any other laboratory animal, explant those tissues, and see what one can learn from the *in vitro* estimates of genotoxicity to make an extrapolation based on actual data from the *in vitro* studies back to the *in vivo* studies, knowing full well that compound X did induce tumors in the animal. To find out whether compound X could induce tumors in the human, the protocol is very simple. One would take the tissues of interest from human donors and do exactly the same types of short-term assays as one can do with the rodent tissues to investigate DNA damage, DNA repair, mutagenesis, or other short-term assays.

The types of tissues which are available for genotoxicity research in short-term assays include most of the epithelial tissues from the human, the tissues which are the sites in the body where most cancer occurs. Therefore, taking epithelial tissues and explanting those tissues or isolating cells from those tissues for use in a variety of short-term tests is useful.

In Curt Harris' review of this topic, he discussed the techniques for explanting the epithelial tissues and indicated that cells isolated from many of these tissues could grow in primary culture in a clonal manner. One obvious exception to that generality is the liver. I don't think that there really is any cell culture methodology for clonal growth of rat or human liver currently available. But the point is that one can make risk assessment from the short-term assays if one can scientifically and logically extrapolate *in vitro* studies back to the *in vivo* situation. The suggestion I will use as a springboard into the discussion for the entire group is that I believe that human cells will be useful for risk assessment purposes if certain criteria are met.

Extrapolation of genetic toxicology data from *in vitro*

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data to *in vivo* would be valid if it can be determined that the routes of metabolism of the chemical are the same *in vitro* as *in vivo*, if the types of DNA adducts produced are the same in culture as *in vivo*, if the cellular defense mechanisms such as DNA repair pathways are the same *in vitro* as *in vivo*, etc. All of these parameters can be investigated to determine the validity of the extrapolation of data from *in vitro* to *in vivo* with animal models.

If it can be determined through investigations with laboratory animals that for a particular chemical carcinogen, the routes of activation and detoxification of the chemical by cells *in vitro* is similar or identical to that observed when the investigations are conducted *in vivo*, and if the spectrum of adducts that one can identify on DNA isolated from a tissue from animals exposed to a particular compound are the same as the types of adducts one finds when cells from that tissue are exposed to the compound in culture, one has established a scientific basis for making the extrapolation from *in vitro* to *in vivo* for that laboratory animal.

To investigate the potential human genotoxicity, one would have to rely heavily on the data from animal models. For example, if it was determined that rats of a certain strain metabolize compound "A" in a manner which results in a certain profile of adducts on hepatocyte DNA, and it was then determined that compound A induced liver tumors in that strain of rat, one could investigate the routes of metabolism and the types of DNA adducts produced on human hepatocytes exposed in culture to compound A. If it were found that hepatocytes, isolated from a number of human cases, metabolized the compound in exactly the same way as the rodent, and the same profile of DNA adducts were produced by rodent and human hepatocytes, one would have a scientific basis for concluding that there is a potential for human genotoxicity from compound A. Whether that potential is ever realized may depend on the exposure of the initiated cells to tumor promoters or other modifying factors. Since cancer is a multistep process, which may involve induction of mutations, chromosomal changes, and the response of initiated cells to tumor promoters, it may be unrealistic to believe that any one short-term *in vitro* assay could faithfully predict the carcinogenicity of all possible chemicals in a whole animal. Just as one cannot measure the activation of protein kinase C with a cyclic-AMP assay, one will not be able to accurately predict the carcinogenicity of chemicals which are not mutagens with a mutagenesis assay. If one simply understands the limitations of each of the short-term assays, one can use that data in a more meaningful way for risk assessment purposes.

I think that the use of human cells for the standard types of genetic toxicology research is intuitively logical. There are certain situations where the short-term assays are going to fall short of being predictive of full carcinogenesis. In particular, human cells may not express the full biological response of exposure to carcinogens, e.g., transformation. This is an area where short-term assays may fall short. However, this is not just true with human cells but with cells from many other species. Many tissues may not be useful for initiation-promotion protocols *in vitro*. And the multistep aspects of carcinogenesis may be very hard to define using only a single-cell type of culture.

The interspecies differences in carcinogen metabolism and DNA adducts have been discussed in several reviews and discussions (*2–5*). But I think that the general consensus is that human cells in general form the same types of metabolites as rat cells. There may be quantitative differences in the amounts of specific metabolites or specific DNA adducts produced by these two species, but not dramatic qualitative differences in the exact types of metabolites that are produced or in the exact types of adducts that are produced. Human cells may also be used for risk assesesment purposes for fine tuning the interpretation of data by asking at a certain level of DNA adducts, Can one expect to observe a genotoxic event? as opposed to simply asking the question, Is this chemical going to be genotoxic in this species?

The last point I'd like to discuss is the multistep area of carcinogenesis and emphasize the newer areas of research, including those involving oncogenes. Activated human oncogenes transform rat cells quite well. Therefore, there may be common pathways to transformation between the species. If one determined that exposure to a particular chemical induced the activation of oncogenes in the liver of a rat, that data may be very relevant to the activation of those same oncogenes in other species, including humans.

However, there are some aspects of carcinogenesis research that can only be addressed with human tissues. Whereas many rat or mouse cells seem to be transformed quite well by transfection of oncogenes, when one puts those same activated oncogenes into human cells, they don't result in the transformation of the cells. There are numerous examples of this in the literature. Experiments have been reported in which mutated *ras* genes, alone or in combination with other cooperating genes such as the *myc* gene, have been transfected into human cells (*6*). The phenotype that results from these experiments with oncogene transfected human cells indicates that the human cells are more difficult to transform with cloned oncogenes than the rodent cells, suggesting that there may be repressor genes in the human cells that antagonize the action of the activated oncogenes. Therefore, the full biological response to oncogene activation in human cells may be slightly different than that observed following the activation of oncogenes in rodent cells. In a situation such as this, one really has to perform studies with human cells and directly determine (possibly on a gene-by-gene basis) which data from experiments performed with rodent cells are going to be directly relevant to humans.

I think at this point I'd like to stop and encourage discussion from the floor. Are there any comments in particular about the use of human cells or reasons why one should not use human cells for carcinogenesis research?

Discussion

DR. LUCIER: Isn't the key part of the process missing? That is, the great difficulty in producing transformations in human cells?

DR. STROM: That's an important point. It's very likely that the full complement of biological responses that one can measure *in vivo* are not going to be reproduced *in vitro* in any short-term test. This is true with short-term tests with rodent cells, also. This problem is not unique to human cells. But I agree with you that the problem is going to be getting complete transformation of cells *in vitro*.

DR. GEORGE LUCIER, NIEHS: Steve, for the kinds of events that hormone or receptor-mediated events might be important to the process of extrapolation, say, estrogen action, and maybe to a lesser extent TCDD action, people who have tried to culture cells, whether it be animal or human, and look at these receptor mediated events (and estrogen is probably the best example) have found that the *in vitro* situation really doesn't at all mimic the *in vivo* situation. The receptor is often lost because you're dealing with a hormonal milieu that's required, that you've taken away, that's required for the maintenance of the receptor, and so forth. For example, the receptor is lost in culture for estrogen in either liver or reproductive tract tissues. So people who have tried to develop *in vitro* models and use them for extrapolating for this or that have really been stymied by this. It might be easier to do for the more genotoxic events. It may be more difficult to do for those events that involve the endocrine system. And I don't know if you care to comment on that, but it's something that people like me who have tried to look at estrogen action *in vitro* have been really blocked by.

DR. STROM: The comment is that cells in culture in general may not be a particularly good model. You're not saying that only human cells are not particularly good in that regard. It's just that the *in vitro* systems themselves do have some shortcomings. Is that the point?

DR. LUCIER: Yeah. And particularly in regards to events relating to the carcinogenic process that are mediated through the endocrine system.

DR. STROM: I think that's a key point. Many of the epithelial tissues have a tendency to dedifferentiate in culture very rapidly and lose some of the differentiated functions that they would have *in vivo*. And that's certainly a problem.

Again, I think understanding the limits of your short-term assay and knowing what you can do with it and what you can't do with it is important. It's very likely that many of those things you brought up are not going to be able to be done in culture until one determines what the appropriate growth factors or substrates are for these cells in culture.

And, again, the problem with the liver, which I'm more familiar with, is that there are certain ways to get around some of these problems. With the liver one can put compounds like phenobarbitol in culture and it seems to maintain the viability of the cells up to many weeks in certain instances. DMSO and coculture techniques, when hepatocytes are maintained in culture with liver epithelial cells, can prolong the differentiated state of these cells in culture for weeks to months. So I think it's very likely that as cell culture techniques are improved and more research is done, that even in the tissues such as the endocrine tissues, one will be able to prolong the viability and differentiated state of many epithelial tissues *in vitro*.

UNKNOWN SPEAKER: Has anybody tried the use of nude animals, that is, immunologically deficient animals, to compare the responses of human tissues as tissues rather than as cells compared to animal cells as a basis for species extrapolation at least of toxic effect?

DR. STROM: Do you mean by transplantation of human tissues into the nude mouse? Is that what you mean?

UNKNOWN SPEAKER: Yes.

DR. STROM: I'm not aware of that being done on any grand scale. There are certain instances where human skin was put on the back of nude mice and someone tried TPA promotion experiments. The situation is that there are certain strains of mice that are easily promoted by TPA and work well on initiation-promotion protocols. Whereas, in other strains of mice, the initiation-promotion protocol with TPA on the back of the mouse skin doesn't work all that well. And when the experiments were done with the human skin, TPA was found to be a weak promoter. I think this points out the fact that work with the human tissues themselves really has to be done even if it isn't in the transplant situation in the nude mouse. Because there are certain differences that one sees between different species and their susceptibility to cancer. And it can only be addressed by using that particular tissue. One can't predict *a priori* what's going to happen unless you do the experiment with human cells or tissues.

DR. BYRON BUTTERWORTH, CHEMICAL INDUSTRY INSTITUTE OF TOXICOLOGY: I think that we've become far too comfortable with our rodent models. We use them a lot. We trust them. We publish all the time. And I think it's very important that what Steve says, that the kind of things that Steve has said get more attention. Let me just give you one example. Bill Greenlee showed me one article, which I think really deserves further consideration. It was an epidemiological study in which 30 years ago, in a plant in Nitro, West Virginia, there was a terrible accident and many people were exposed to TCDD, 121 people. They all had chloracne, and beyond that they had severe problems such as liver enlargement, liver tenderness. And it lasted for more than 4 years.

Thirty years later they did a follow-up epidemiological study, and there was no increase in the incidence of cancer. Which raises the very, very frightening question, Why is a chemical that is so extraordinarily potent in the rodent not an obvious human carcinogen? And the disturbing companion question is, Are there potent human carcinogens that we're not recognizing because

they're negative in the rodent model? So when we asked before what is the importance of determining what the mechanism is—is it cell proliferation, is it promotion via receptors—I think it's very important because these kinds of things may be the very points that will help clarify the differences between species specificity. So looking both at genotoxic events, metabolic activation, and going beyond that looking at hormonal responses, I think it's very, very important for us in the real world of risk assessment.

DR. MARSHALL ANDERSON, NIEHS: I hear what you're saying. But is that really a fair comparison? Because I don't think the animal studies being done, like with Henry Pitot's model, or in the skin with TCDD where you have come in with just one high dose. I think in all of those studies it has been continuous low-level exposure. I hear what you're saying, and I think it would be interesting to go back. So tell Bill Greenlee that he should do that experiment to go back with Pitot's model. What are you shaking your head about?

DR. ANDERSON: Yeah. But they were spread. It was one dose every 2 weeks. It was still spread over a long time. Right?

UNKNOWN SPEAKER: Right; now he's doing it for 1 year. So 26 doses every other week is the dose. And they're subcutaneous doses, so it's a very gradual release. So it's a chronic exposure situation.

UNKNOWN SPEAKER: That's right. The effect went away. But I would add one other thing to that comment regarding TCDD. In the NTP study in the Kociba study, I believe liver tumors were only seen in female rats. Is that right? And the individuals in West Virginia, were they mostly males?

UNKNOWN SPEAKER: But in the rat. In the most it was a little bit different. In the rat, the Kociba study and the NTP study exhibited liver tumors only in the male animals.

UNKNOWN SPEAKER: I'm sure. Is he still here? Dr. Kociba? That's too bad. But in any event, there was a clear sex difference and it was reversed in the mice, the liver tumors. And the point is that it relates back to the *in vitro* studies that, in addition to other kinds of things, we have to look at the sex difference as well. And that relates to something I'm interested, of course, the endocrine differences in the extrapolation process of reconstituting those kind of systems where I think we have to be concerned about sex difference as well as responsiveness.

DR. STROM: I think one of the key points that I've heard today over and over again now is: Is an *in vitro* assay predictive of *in vivo* genotoxicity? I think we can turn that around a little bit for purposes of this discussion and say, Are *in vivo* toxicity studies done with one species predictive of the next species? And I think I'd like to ask Dr. Tennant that if I might.

As I recall, 50 to 70% of the chemicals that were identified as mutagens in some sort of an *in vitro* study end up being carcinogens in the whole animal in one species or another. Then one takes that data and asks, What is the correlation between carcinogenicity in mice and rats?

DR. TENNANT: 75%.

DR. STROM: Okay. So it does improve a bit. So basically, if one uses as the gold standard the *in vivo* bioassay, one is still left with the interspecies extrapolation that only improves about 20% from the *in vitro* studies. And when one comes up with a compound that can induce tumors in mice or rats and we're trying to extrapolate it to humans, we have to determine whether we are mice or rats. And some of the ways, as Dr. Butterworth has said, that this might be done is to look at the mechanistic approach. Do they make the same metabolites? Do they make the same adducts? Are they repaired at the same rate? And so on, with everything that may affect the carcinogenicity of the compound, including cell turnover. Experiments can then be done to address these points to make the interspecies extrapolation. But, again, the interspecies extrapolation is probably only as good as the data that one can get in the short-term assays.

DR. ALBERT: We'll move on to the next area, which is, How do we predict toxicity of complex mixtures?

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